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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project is to delineate the potential role(s) of excitatory amino acids (EAA) as mediators of central nervous system (CNS) excitation and seizures produced by centrally-active cholinomimetic agents and to evaluate possible palliative treatments for central cholinomimetic toxicity. The scope of this project entails simultaneous use of neurochemical and electrophysiological approaches that are designed to assess cholinomimetic-induced excitation in the rat CNS. The goals for this report period were: (1) to assemble a new on-line microdialysis instrument for rapid measurement of L-glutamate (GLU) and L-aspartate (ASP) in brain extracellular fluid, (2) to characterize the regulation of EAA levels by metabotropic GLU autoreceptors, and (3) to undertake preliminary studies of EAA levels in CNS tissues of cholinomimetic-treated rats. The first goal has been achieved insofar as the instrument has been assembled and validated in vivo. The second goal has been achieved and results demonstrate profound differences between striatum and hippocampus with respect to the efficiency of metabotropic GLU autoreceptor control over EAA levels. On-going studies related to the third goal have revealed that cholinomimetic agents produce differential and time-dependent changes in GLU and ASP levels in these brain regions. As planned, these studies will continue during year two.

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A. INTRODUCTION

This project is designed to evaluate the role of excitatory amino acids (EAA) in the brain as possible mediators of central nervous system (CNS) toxicity that is caused by exposure to the organophosphate (OP) class of acetylcholinesterase (AChE) inhibitors as well as other centrally-active cholinomimetic agents. While OP agents cause profound CNS stimulation, including seizures, convulsions and irreversible neuropathological damage, current treatments to protect against or reverse other symptoms of OP poisoning fail to ameliorate these CNS toxicities. Current evidence implicates glutamate (GLU) and possibly other excitatory amino acids (EAA) in the excitatory and pathophysiological actions of centrally-active cholinomimetics. The overall goals of this project are to critically evaluate the putative role of GLU as a mediator of the central excitatory effects of these agents and to measure directly the ability of metabotropic GLU autoreceptor ligands to protect against and/or reverse the central actions of cholinomimetic agents. The overall approach involves simultaneous measurements of electroencephalographic (EEG) activity and neuronal release of GLU and L-aspartate (ASP) in rats. The latter measure will be carried out with a novel procedure that affords an enhanced capacity to measure extracellular GLU and ASP *in vivo* with a high degree of temporal resolution. By combining this novel technology with simultaneous electrophysiological correlates of neuronal activity (EEG measurements), it should be possible to establish the role of GLU as a mediator of CNS excitation by cholinomimetic agents and to demonstrate the utility of metabotropic GLU receptor ligands as a means to prevent OP-induced seizure activity.

B. BODY

The major tasks accomplished during this reporting period were: (1) the assembly of a new instrument for on-line measurements of GLU and ASP by *in vivo* microdialysis coupled with separation and detection of analytes by capillary electrophoresis and laser-induced fluroescence, respectively; (2) characterization of neuronal release of excitatory amino acids from hippocampal neurons during electrical stimulation of the perforant pathway and evaluation of the regulatory influence by metabotropic GLU autoreceptors; and (3) a preliminary evaluation of the effects by direct and indirect-acting cholinomimetic agents on GLU and ASP levels in the striatum and hippocampus of anesthetized rats. Detailed summaries of experimental results are provided in the following sections. Relevant experimental data are summarized in figures and charts that are located in Appendix I.

1. Assembly Of A New Instrument For Fast On-Line Monitoring Of Amino Acid Neurotransmitters In Anesthetized Rats Using In Vivo Microdialysis.

As described in our original proposal, a major objective of the first year of this project was to design, construct, and characterize a new microdialysis-capillary electrophoresis (CE)-laser induced fluorescence (LIF) instrument for the *in vivo* monitoring of neurotransmitter dynamics. This portion of the project has proceeded successfully. In the course of building this instrument, we were able to make substantial improvements in the selectivity and sensitivity relative to the first generation instrument used to obtain preliminary data shown in the grant application. The new instrument will allow monitoring of more neurotransmitters along with GLU and ASP. In addition, the added sensitivity will facilitate more precise

measurements of the excitatory amino acids. The following sections describe our progress in this objective.

The design of the new microdialysis-CE-LIF instrument that was constructed this year is shown schematically in Figure 1.1. Neurotransmitters are extracted from the brain region of interest using a microdialysis probe. The primary amines are reacted with *o*-phthalaldehyde (OPA) on-line to form fluorescent isoindoles. Narrow plugs of the derivatized amines are injected onto the separation capillary using a flow-gating interface. The derivatized neurotransmitters are separated using CE and detected using LIF in a high-sensitivity sheath-flow detection cell. A more detailed description of the instrument operation and characterization are given in the following sections.

On-line Derivatization. An important step in our analytical procedure is the on-line derivatization of dialysate. Experiments were performed to optimize this step for sensitivity and quantification. The optimized conditions are described here. Dialysate is pumped from the sampling probe at 0.3 μL/min through a 75 μm inner diameter (I.D.) x 360 μm outer diameter (O.D.) fused silica capillary where it is mixed with a 0.3 μL/min stream of derivatization solution consisting of 10 mM OPA, 40 mM β-mercaptoethanol (BME), 40 mM borate buffer at pH = 10.5. The dialysate and derivatization solution are mixed and reacted in a 15 cm length of 75 μm I.D. fused capillary under continuous flow. OPA is a fluorogenic reagent that reacts with primary amines in the presence of nucleophiles to form fluorescent isoindoles. OPA derivatized amino acids are fluorescent with excitation and emission wavelengths of 350 nm and 450 nm, respectively. An advantage of this reaction scheme is the speed of the reaction, which is quantitative in less than 20 s.

Flow-Gate Injection Interface. After on-line derivatization, the analytes must be injected in narrow plugs onto the CE capillary. The injections must be automated and allow highly efficient separations. To perform these injections, we used a modification of the flow-gated interface used in our first generation instrument (See Figure 1.2). In the interface, the outlet of the reaction capillary is aligned with inlet of the CE separation capillary, leaving a gap of approximately 30 μm. A 1 mL/min cross-flow is applied between the capillaries which carries the sample solution (derivatized dialysate) to waste. To inject a plug of sample, the separation voltage is reduced to zero (3 s) and then the cross-flow is stopped for 1 s using a solenoid valve. This maneuver allows sample to cross the gap between the capillaries. The voltage is then raised to 2 kV for 200 ms to inject a plug of dialysate onto the separation capillary. After injection, the gating cross-flow is resumed and the separation voltage was ramped up to 20 kV over 500 ms. The flow-gate is held at ground to isolate the animal from the voltage dropped across the separation capillary. All of these switches are controlled by a PC and data acquisition and control board using a program written in LabView (National Instruments, Austin, TX) in our laboratory.

Capillary Electrophoresis (CE). The CE separation conditions used with the new instrument have been modified to allow for the simultaneous determination of GABA, dopamine, glycine, GLU and ASP. CE is carried out in a 10 cm long, 10 μm I.D. fused silica capillary. A separation voltage of -20 kV is applied at the outlet of the separation capillary. The electrophoresis buffer is 40 mM borate / 1mM β-cyclodextrin adjusted to pH = 10.5 using 1 M NaOH. β-cyclodextrin forms a host-guest complex with GABA which adjusts is migration rate sufficiently to separate it from other singly charged derivatives of primary amines typically found in the brain.

Sheath-Flow Cell / Laser Induced Fluorescence (LIF). In the new instrument, the OPA-labeled amino acids are detected using LIF in a high-sensitivity sheath-flow detector cell (Cheng and Dovichi, 1988) as opposed to the on-column system used in the first instrument. As shown in Figure 1.2, the outlet of the separation capillary is inserted into a 2 mm × 2 mm square O.D. by 200 μm × 200 μm square inner bore fused-silica cuvette. Separation buffer is siphoned around the outside of the outlet of the separation capillary using gravity flow (15 cm difference between buffer reservoir levels). Analytes migrate off the end of the separation capillary with a laminar flow profile. Analytes are detected within this laminar flow using LIF. This arrangement reduces the background signal caused by laser scatter, which usually limits sensitivity in on-column detection. Fluorescence is excited using the 351 nm line (20 mW total UV) of an argon-ion laser focused onto the analytes using a 1× fused silica lens. Emission is collected at 90° using a 60×, 0.7 N.A. long-working distance objective, spatially filtered using an iris, passed through a 450 nm bandpass filter and collected on a photomultiplier tube (PMT). Photocurrent from the PMT is amplified and filtered (10 ms rise-time) using a current-amplifier and collected using a data acquisition board and a PC.

1.3 Characterization of Instrument Performance

The significant improvements in the new instrument relative to the first-generation instrument are: 1) improved sensitivity by incorporation of a sheath-flow cuvette to reduce noise, and 2) improved separation to allow detection of more neurotransmitters. While incorporating these improvements took longer than simply building a twin of our first-generation instrument, we believe that these improvements will greatly increase the functionality of the new instrument and allow more comprehensive studies of the cholinomimetic agent effects in vivo. A description of the characterization of the new instrument is given in this section.

Improved sensitivity. The sheath-flow cuvette improves the signal to noise ratio of a measurement by reducing the background signal caused by laser scatter. Laser scatter often sets the limit of detection in on-column detection. Figure 1.2 demonstrates how laser scatter is reduced. Light is scattered whenever there is a refractive index change in the medium that it is passing through. In on-column detection this occurs at the inner and outer walls of the capillary. Because the capillary is round, scatter is reflected in 360^0, resulting in a significant background signal. In the sheath-flow cuvette, the only refractive index change occurs at the walls of the cuvette which is 100 μm from the fluorescent signal. The distance from the signal allows the background scatter to be removed by spatial-filtering. Using separation conditions similar to those used previously, we have been able to achieve limits of detection of 1 nM for GLU and ASP. This represents a 15-fold improvement over our previous results and is much lower than the levels typically found *in vivo* (1 μM and 500 nM for GLU and ASP, respectively; Herrera-Marschitz et al., 1996).

Analysis of Additional Neurotransmitters. Although our previous protocols have been very successful in monitoring GLU and ASP *in vivo*, this only shows part of the picture. There are other important primary amine neurotransmitters, including glycine, dopamine and GABA that could be involved in the actions of cholinomimetic drugs. It would be advantageous to monitor all of these analytes simultaneously, allowing interactions between the individual neurotransmitters to be determined.

There are two issues that need to be addressed to develop a simultaneous separation for GABA, glycine, dopamine, GLU and ASP: 1) efficiency and 2) selectivity. The separation in our current microdialysis-CE-LIF methodology has an efficiency of 30,000

theoretical plates for GLU and ASP. (Theoretical plates are a measure of separation performance or efficiency. The number of theoretical plates is given by t_{mig}^2/σ^2 where t_{mig} is the migration time and σ is the zone width expressed as a standard deviation. Thus, high plates correspond to narrow zones.) This number of plates is lower than that typically achieved in CE. It was determined in our experiments that the low number of plates was due to injection of overly large plugs of sample in the first instrument. The large injections were done to improve detection limits. The improvements in sensitivity achieved using the sheath-flow detector has allowed us to decrease the amount of dialysate injected for separation. In addition, it was found that the ramped voltage injections (see section 1.2) and higher electric field both allowed further improvements in separation efficiency to 500,000 plates (≈30 ms peak widths). The maximum peak density at this efficiency is high enough to allow the simultaneous analysis of many analytes even in very short separation times.

The other issue that we have addressed to optimize the separation of neurotransmitters is selectivity. Migration time in CE is determined by the charge to size ratio of the analyte. This is the reason that GLU and ASP are so well resolved from the other peaks. The OPA derivatives of GLU and ASP are one of the few doubly charged analytes present in the dialysate. Conversely, there are many singly charged OPA derivatives, making separation of these analytes difficult. Of particular concern is GABA, which migrates near the middle of the band of singly charged OPA derivatives found in brain dialysates. To overcome this problem we have added β-cyclodextrin to the separation buffer to modify the selectivity of separation. Cyclodextrins have a hydrophobic cavity that can form a host-guest complex with analytes of appropriate size. The migration time of analytes are know determined by the equilibrium with the cyclodextrin as well as its charge and size.

Figure 1.3 compares how GABA and a generic amino acid are expected to interact with β-cyclodextrin. The steric effect caused by the branch at the α position of the isoindole formed by the amino acid decreases the binding efficiency of the cyclodextrin complex. GABA does not have this steric effect and is expected to bind β-cyclodextrin better. We therefore hypothesized that at low concentrations of β-cyclodextrin, GABA would bind much better than the other amino acids present in the dialysate, shifting the migration time of GABA ahead of the peaks for the other singly charged OPA derivatives. Figure 1.4 shows this hypothesis to be correct. At low concentrations of β-cyclodextrin, GABA migrates with the other singly charged derivatives. As the β-cyclodextrin concentration is increased, GABA pulls ahead of the other analytes allowing baseline resolution.

Figure 1.4 demonstrates the separation power possible with the improved conditions. Baseline separations are achieved for dopamine, GABA, glycine, GLU and ASP which will allow interactions between these neurotransmitters to be monitored *in vivo* for the first time on a relatively fast time scale. Such measurements will be invaluable in discerning the effects of cholinomimetics on neurotransmission and seizurogenesis in the forebrain.

Testing of the new instrument by in vivo measurements. Having demonstrated significant improvements over the previous instrumentation we have begun *in vivo* testing of the new instrument. Figure 1.5 shows electropherograms obtained on-line under basal conditions for dialysate collected from the rat striatum. As in the previous protocol, GLU and ASP are well resolved. Glycine appears to be well resolved as well. GABA is not completely resolved; therefore, further optimization of the separation is planned by adjusting the β-cyclodextrin concentration, buffer pH, and borate concentration. Even without this optimization however, the resolution of GABA is sufficient for quantification. Basal levels of

dopamine were not detected in these initial experiments. This is not unexpected as basal levels for dopamine have been reported to be lower than our limit of detection (≈1 nM).

Figure 1.5A shows the in vivo neurotransmitter levels during stimulation with 145 mM KCl. Clearly, there were large increases in the extracellular concentrations of GLU, ASP and GABA. Glycine also showed a moderate increase. Dopamine was also detected during the stimulation. Figure 1.6 is a contour plot that demonstrates the temporal variation of various peak heights during the KCl stimulation. Again increases are observed for dopamine, GABA, glycine, GLU, and ASP. The resolution possible with this measurement also reveals that several other peaks, as yet unidentified, actually decrease during the stimulation. In addition, other peaks increase but only after the stimulation has been completed. These variations demonstrate the ability of this technique to monitor interactions between the various analytes present in the extracellular fluid.

Concluding Remarks. With the completion of this instrument, we now have two fully functioning instruments for monitoring neurotransmitters in vivo in the coming two years. The new instrument has improved capabilities over the old instrument allowing monitoring of *all* of the major amino acid neurotransmitters plus elevated levels of dopamine. While the development of this second instrument required more time than we had previously allotted for instrument building, these improved capabilities will allow a much better picture of the neurotransmitter dynamics underlying seizurogenic actions of cholinomimetics than what was possible previously. Even as this instrument was being built however, we used the already developed, first generation instrument, to begin our studies of GLU regulation in the forebrain by metabotropic GLU receptors. These studies are described in the next section.

2. ***Ionic Requirements And Metabotropic Autoreceptor Regulation Of Electrically-Stimulated GLU And ASP Release From Hippocampal Neurons In Vivo.***

As described in our original proposal, the second and third objectives for year one of this project involved preliminary studies of the effects by cholinomimetic agents on EAA levels in representative brain regions and validation of our method for measurement of EEG activity in anesthetized rats. Before undertaking part one of this objective, it was necessary to validate our microdialysis procedure in a brain region that is associated more closely with the seizureogenic actions of cholinomimetic agents. For this purpose, we selected the hippocampal formation as our initial region to study. While these studies revealed that neuronal GLU release could be detected within the hippocampus, it was evident that the control of release by metabotropic autoreceptors was dissimilar from the striatum (see below). Since this finding was contrary to our initial expectations, additional studies were carried out in order to characterize the pharmacology of hippocampal metabotropic receptors. Although these experiments were projected for Year Three of this project, it was necessary to carry out selected studies at this time in order to facilitate a meaningful interpretation of the disparate findings between the hippocampus and striatum. In view of the substantial efforts on this portion of the project, studies of EEG activity following treatments with cholinomimetic agents have been moved back and are now the top priority for Year Two of the project.

Microdialysis Studies In The Striatum. Our previous work in the chloral hydrate-anesthetized rat model involved a detailed characterization of neuronal GLU release within the corticostriatal pathway (Lada et al., 1998). In that study, we were able to demonstrate for the first time that it is possible to monitor selectively the neuronal release of GLU and ASP *in vivo* by using microdialysis in combination with the instrumental setup and analytical

procedures described above. Two experimental observations provided strong support for this conclusion. First, it was noted that the rapid rise in extracellular levels of GLU and ASP within the striatum was entirely dependent upon the normal operation of tetrodotoxin (TTX)-sensitive voltage-gated sodium channels following brief electrical stimulation of the prefrontal cortex. In addition, the stimulus-dependent rise in striatal EAA levels was prevented completely by localized depletion of extracellular Ca^{2+}. Together, these results were consistent with the hypothesis that measurement of stimulus-dependent EAA release in this model could provide an accurate indicator for synaptic transmitter release. This view was substantiated further by several other experimental observations. Infusion of the GLU transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (200 μM, PDC) increased resting GLU and ASP levels by 300 to 500% without altering electrically-stimulated increases in GLU or ASP concentrations. However, in the presence of the metabotropic GLU receptor (mGLUR) agonist (1S,3R)-1-aminocyclopentane-trans-1,3-dicarboxylate (200 μM, ACPD), stimulated increases in GLU and ASP concentrations were suppressed by 91% and 73%, respectively. This effect was blocked by the mGLUR antagonist (RS)-α-methyl-carboxyphenylglycine (200 μM, MCPG). While MCPG alone produced no change in electrically-stimulated release of GLU and ASP, this antagonist produced a five to six-fold increase in stimulated overflow when tested in the presence of the transport inhibitor PDC. Based upon these results, it was concluded that release of GLU and ASP from corticostriatal neurons could be measured by *in vivo* microdialysis and that synaptic GLU release could be regulated through the administration of exogenous drugs which interact selectively with metabotropic GLU autoreceptors. It was postulated that this could be an important mechanism for controlling excitatory neurotransmission at synapses in the corticostriatal pathway as well as other forebrain pathways that utilize GLU and/or ASP as the primary synaptic transmitters.

Characterization of EAA Release In The Hippocampus. While these experimental results provided compelling support for our use of *in vivo* microdialysis coupled with instrumentation for fast time-scale measurements in order to monitor neuronal EAA release, it was important to determine the extent to which results from studies in the striatum were representative of other glutamatergic forebrain pathways. As outlined in the original proposal, there are several significant reasons behind our decision to focus studies of cholinomimetic effects on GLU-mediated synaptic transmission in the hippocampus. In view of this, we developed and carried out an experimental protocol for monitoring the release of GLU and ASP from excitatory hippocampal terminals. As shown in Figure 2.1, brief electrical stimulation of the perforant path produced a modest rise in extracellular GLU and ASP levels in the hippocampus. In comparison to stimulus-dependent changes observed in the striatum (Lada et al., 1998), the increases measured in hippocampus were substantially smaller in magnitude and of shorter duration for both GLU and ASP (Figure 2.1). However, the diminished response in hippocampus does not appear to be related to the efficiency of fiber activation within the perforant path insofar as stepwise changes in the stereotaxic placement of the stimulating electrode failed to elicit any significant improvement in stimulus-evoked changes (data not shown). Additional studies were undertaken to ascertain the extent to which the stimulus-dependent rise in hippocampal GLU levels was derived from neuronal transmitter release. As shown in Figure 2.2, local intracranial infusion of TTX by reverse microdialysis caused a complete inhibition of stimulus-evoked GLU release. Since TTX is a potent and selective inhibitor of voltage-activated neuronal sodium channels, this

result provides support for the view that the stimulated rise in hippocampal GLU concentration is neuronal in origin. This view is strengthened by the independent observation that the stimulated increase in GLU concentration is a calcium-dependent process. Localized depletion of extracellular calcium was achieved by perfusing the probe with a calcium-free artificial cerebrospinal fluid containing EGTA. This protocol has been used successfully in rat striatum to ablate the response to electrical stimulation (Lada et al., 1998). Under these conditions, the response to perforant path stimulation was decreased by more than 80 percent in the hippocampus (Figure 2.3). Thus, in view of the marked inhibitory effects produced by TTX or calcium ion depletion, it is evident that the increase in extracellular hippocampal GLU levels associated with perforant path stimulation represents an indirect measure of synaptic GLU overflow. In this regard, the basic nature of stimulus-evoked changes in GLU levels appear to be qualitatively similar between the hippocampus and striatum.

Metabotropic Autoreceptor Regulation of Hippocampal GLU Release. While the ionic requirements for GLU release appear similar for hippocampal and striatal neurons, additional studies were undertaken to ascertain the independent roles and possible interplay between high-affinity EAA transporters and metabotropic GLU autoreceptors in the hippocampus. Direct intracranial infusion of a potent and selective inhibitor of EAA transport (PDC) produced a dramatic five- to six-fold rise in the basal GLU level and a remarkable ten-fold increase in stimulated GLU overflow when compared to drug-naive control rats (Figure 2.4). When compared to the striatum, the absolute magnitude of changes produced by PDC treatment is significantly greater in hippocampus. Since the consequences of EAA transport inhibition are substantially greater in hippocampus than striatum, it seems likely that GLU removal from the extracellular compartment is more efficient in the former region. In retrospect, the more efficient removal of extracellular GLU via cellular transport in the hippocampus may explain the relatively small and short duration of the increase in extracellular GLU levels following perforant path stimulation (Figure 2.1). However, it is also possible that the blunted response could be a direct consequence of a highly efficient tonic control of synaptic GLU release in hippocampus by presynaptic inhibitory metabotropic autoreceptors. Since the functional role of these hippocampal autoreceptors during cholinomimetic-induced seizures is a major focus of this project, it was important to characterize the overall efficiency of metabotropic autoreceptor-mediated control of impulse-dependent GLU release under normal conditions. As shown in Figure 2.5, blockade of metabotropic receptors with the potent antagonist MCPG produces a modest increase in the basal GLU level as well as a somewhat larger increase (149% of control) in the stimulus-evoked rise in hippocampal GLU. Infusion of the non-selective metabotropic receptor agonist ACPD causes a small reduction in basal GLU levels with no substantial change in the stimulated response (Figure 2.6). Taken together, these results provide evidence for the presence of inhibitory metabotropic autoreceptors which serve to control activity-dependent neuronal GLU release in the hippocampus. This represents an important finding insofar as a major premise for this project was the existence of these autoreceptors in areas of the brain which are strongly affected by cholinomimetic agents. However, it is important to note that these preliminary results indicate a functional difference between metabotropic autoreceptors in the hippocampus and striatum. As outlined above, previous work in this laboratory has demonstrated that metabotropic autoreceptors in striatum provide an inhibitory mechanism for feedback control of stimulus-dependent GLU release under a variety of conditions. However, this does not appear to be true for hippocampal metabotropic autoreceptors. As shown in

Figure 2.5 and 2.6, the autoreceptor antagonist MCPG exerts opposite effects on stimulated GLU release when tested alone (facilitates release) or in the presence of PDC (inhibits release). Since the effects of MCPG should derive entirely from blockade of autoreceptor stimulation by endogenous GLU, it would appear that metabotropic autoreceptors provide feedback inhibitory control over GLU release when the extracellular level of GLU is relatively low. However, in the presence of markedly elevated levels of endogenous GLU (i.e., when PDC is present), MCPG-sensitive autoreceptors appear to operate as a facilitative feed-forward mechanism for controlling GLU release. While, at this time, we have no definitive explanation for these seemingly divergent results, a recent report by Rodriguez-Moreno and coworkers (1998) provides a clear precedent for a use-dependent switch in the functional role of hippocampal metabotropic autoreceptors. Since it is anticipated that centrally-active cholinomimetic agents will cause a prolonged activation of glutamatergic neurons with a subsequent rise in extracellular GLU levels, a thorough understanding of this speculated use-dependent reversal of autoreceptor function is important to our overall goal of controlling the excitotoxic actions of these agents.

3. Preliminary Studies Of Effects By Direct And Indirect-Acting Cholinomimetic Agents In Striatum And Hippocampus.

Although the efforts to characterize metabotropic autoreceptor function in the hippocampus are not yet complete, it was necessary to undertake preliminary studies of the effects produced by cholinomimetic agents in our animal model. This undertaking represents the final goal listed for the Year One in our Statement of Work and is an effort that is projected to continue throughout the second year of this project.

Systemic Administration Of Physostigmine. As outlined in our original experimental design, we proposed the use of direct as well as indirect-acting cholinomimetic agents in these studies. The first agent that was tested in our animal model was physostigmine, an indirect-acting cholinomimetic agent which is a potent inhibitor of acetylcholinesterase and several related hydrolytic enzymes. For these studies, physostigmine was administered via intramuscular injection (0.3mg/kg) and levels of GLU and ASP were monitored in the hippocampus and striatum. Experimental results with physostigmine have been variable and possibly confounded by the systemic toxicities of this agent. Therefore, we have decided to focus our initial efforts on the actions of pilocarpine, a direct-acting cholinomimetic agent which can be administered locally via intracranial infusion at or near the site(s) of interest.

Intracranial Administration Of Pilocarpine. Pilocarpine was considered to be an excellent prototype agent in view of its capacity to produce seizures following intracranial administration in rats (Millan et al., 1993; Smolders et al., 1997a,b,c) and the added advantage of avoiding many of the peripheral adverse effects associated with systemic drug administration. Results from our studies with pilocarpine are shown in Figure 3.1. Continuous intra-hippocampal infusion of pilocarpine (10mM) produced remarkably different effects on extracellular levels of GLU and ASP. Immediately following the infusion of pilocarpine, hippocampal ASP levels decreased by nearly 50 percent and remained at this level for the remainder of the experiment. However, as shown in the top panel of Figure 3.1, extracellular GLU levels exhibited a different profile. Immediately following the infusion of pilocarpine, GLU levels decreased by 35 to 40 percent for a period of 10 to 15min. Thereafter, despite the on-going infusion of pilocarpine, GLU levels increased to greater than 200% of the pre-drug level. This increased concentration of GLU in extracellular fluid was

maintained for the duration of the experiment (2 hours) and persisted even after the discontinuation of pilocarpine infusion. These observations are important for the following reasons. First, the marked disparity between GLU and ASP levels following pilocarpine infusion argues against a possible contribution by any disruption in the activity of EAA transporters. Since GLU and ASP are considered to be excellent and nearly equivalent substrates for EAA transporters located on neurons and glia, it is difficult to envision a scenario whereby altered transporter activity could account for the observed changes. Consequently, the biphasic changes in GLU levels and the decline in ASP levels are likely to reflect alterations in the release of these EAA from tonically active neurons. A second important aspect of these results involves the biphasic changes in GLU levels during continuous pilocarpine infusion. In our original application, we proposed that a delayed rise in GLU levels could occur following anti-cholinesterase poisoning and, that this latent involvement by GLU could be responsible for the lack of efficacy by anti-cholinergic drugs during later stages of cholinomimetic-induced seizures. Therefore, while these results are still preliminary, they fit well with our proposed model and afford us the opportunity to evaluate the potential utility of metabotropic autoreceptor ligands as treatments for the seizureogenic actions and convulsant properties of cholinomimetic agents.

C. KEY RESEARCH ACCOMPLISHMENTS

- development of an improved system for monitoring of neurotransmitters *in vivo* based on microdialysis coupled with capillary electrophoresis (CE) and laser-induced fluorescence (LIF) detection. This system allows simultaneous monitoring of GLU, ASP, GABA, glycine, and dopamine with approximately 15 sec temporal resolution. Several important instrument developments were required to achieve this accomplishment, including the development of a high-sensitivity sheath-flow LIF cell for detection, the development of an appropriate instrument control to allow injection onto the electrophoresis capillary, and the development of electrophoresis buffer that allowed the appropriate separation. No other instrument has been developed that has this combination of multiple neurotransmitter measurement and temporal resolution.

- demonstration of neurotransmitter overflow in the hippocampus and striatum following electrical stimulation. Previous measurements of GLU and ASP in the brain by microdialysis have not allowed detection of neurotransmitter dynamics that had the characteristics expected for neurotransmitter release. Our fast time-scale measurements have allowed observation of TTX-sensitive, Ca2+-dependent release which is considered proof of a neuronal origin for these excitatory transmitters. These results indicate that we can "access" neurotransmitter pools for release studies in both the hippocampus and the striatum.

- identification of important control points in the overflow of GLU in the striatum and hippocampus. Specifically, in the striatum, overflow is regulated by mGLUR and uptake. In the hippocampus, uptake dominates the regulation of overflow. mGLUR autoreceptor regulation is weak in the hippocampus and follows an atypical pattern. Specifically, at elevated levels mGLUR antagonists suppress release. This suggests a potentially novel approach to regulation of GLU. These results also have very interesting impliciations for

the role of GLU as a neurotransmitter in vivo. Specifically, the very strong uptake in the hippocampus may imply minimal overflow from synapses and little or no volume transmission by GLU under normal conditions. In contrast, obvious overflow of GLU in the striatum suggests the potential for volume transmission of GLU in this brain region. This may also suggest a lower tolerance for elevated GLU in the hippocampus than in the striatum.

- identification of pilocarpine as a suitable cholinomimetic which alters GLU levels in a manner that is consistent with GLU-evoked seizures. Preliminary studies have revealed physostigmine to be a less suitable agent for these applications in view of its inability to alter GLU levels following intracranial administration and the pervasive physiological effects that were produced following systemic administration of this drug.

D. REPORTABLE OUTCOMES

Reportable outcomes for this period include three abstracts (see below) which resulted in three separate presentations at international meetings.

- Kennedy, R.T., Witowski, S., Thompson, J. and Boyd, B. (1999) Capillary electrophoresis and capillary chromatography for *in vivo* monitoring or amino acid and peptide neurotransmitters. *Proceedings Of The 8th International Conference On In Vivo Methods: Monitoring Molecules In Neuroscience.* June 19-23.

- Witowski, S.R. and Kennedy, R.T. (1999) Monitoring neurotransmitter amino acids by microdialysis with on-line flow gated capillary electrophoresis. *Proceedings Of The 8th International Conference On In Vivo Methods: Monitoring Molecules In Neuroscience.* June 19-23.

- Kennedy, R.T., Vickroy, T.W., Witowski, S.R., Thompson, J.E., Boyd, B. and Phillips, I. (1999) Monitoring amino acid neurotransmitters in vivo with high temporal resolution using microdialysis. *Neuroscience Abstr.* 25: 2232.

E. CONCLUSIONS

The results of work conducted during the first year are exciting and continue to hold strong promise for identifying a useful strategy to treat brain seizure activity associated with cholinomimetic intoxication. Among the key research achievements during the past year (see Section C), three independent results hold particular significance. Foremost among the significant accomplishments was the development of a CE-LIF detection system with capabilities that exceed our initial projections. With this unique instrument, it will be possible to monitor simultaneously the extracellular levels of more transmitters *in vivo* while, at the same time, maintaining excellent temporal resolution. The expanded capabilities of this instrument will benefit the current project as well as numerous other investigations that require fast sensitive neurochemical measurements in living animals.

In addition to the successful development of this state-of-the-art detection system, two significant biological findings have been made. First, our preliminary data provide strong evidence that metabotropic autoreceptors in hippocampus exert a functional control over GLU release in a manner that differs substantially from metabotropic receptor-mediated regulation in the striatum. This discovery is novel and significant insofar as the type of metabotropic receptor ligand that may be needed to treat seizures (*i.e.*, agonist *vs.* antagonist) could depend on whether the agent was used as a prophylactic or as an antidotal treatment. Although our working model is highly speculative at this time, our preliminary results from hippocampus indicate that metabotropic autoreceptors undergo a functional switch following exposure to high extracellular GLU levels. Therefore, prior to the onset of seizures and elevated extracellular GLU levels, metabotropic autoreceptors are inhibitory and would most likely reduce neuronal GLU release when occupied by an agonist. However, following some period of exposure to elevated concentrations of GLU, it appears that metabotropic autoreceptors undergo a functional switch and become facilitatory for neuronal GLU release. Consequently, following the onset of seizures and elevated GLU levels, an antagonist drug may be effective in diminishing additional GLU release. Obviously, a more thorough understanding of this regulatory mechanism is needed before it becomes feasible to evaluate the potential medical applications of this information.

The final significant result from our studies is, at present, the least understood. We have determined that intracranial administration of pilocarpine, a seizureogenic cholinomimetic agent, produces a highly unusual profile of changes in extracellular EAA levels. Following pilocarpine infusion, extracellular levels of GLU and ASP show a dramatic decline in hippocampus. While ASP levels remain suppressed throughout pilocarpine treatment, GLU exhibits a remarkable time-dependent increase that persists well beyond the period of drug treatment. These observations are highly atypical insofar as they demonstrate a clear difference in the effects of a single drug treatment on the two principal EAA transmitters in the mammalian CNS. Such a difference between GLU and ASP is unprecedented. In addition to this novel finding, the prolonged and persistent rise in GLU levels is consistent with the development of a "feedforward" control mechanism which, once activated, promotes an on-going release of neuronal GLU. While it is impossible to ascertain the scientific or medical significance of these observations at this time, the results are considered to be important in view of their disparity with current hypotheses regarding excitatory synaptic transmission in the CNS.

F. REFERENCES

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Lada, M.W., Vickroy, T.W. and Kennedy, R.T. (1998) Evidence for neuronal origin and metabotropic receptor-mediated regulation of extracellular glutamate and aspartate in rat striatum in vivo following electrical stimulation of the prefrontal cortex. *J. Neurochem.* 70: 617-625.

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G. APPENDIX I (Figures)

The figures on the following pages (17 – 29) are related to experimental results that are summarized above in the BODY of this report (Section B). The figures are arranged in the same order in which they are discussed in Section B.

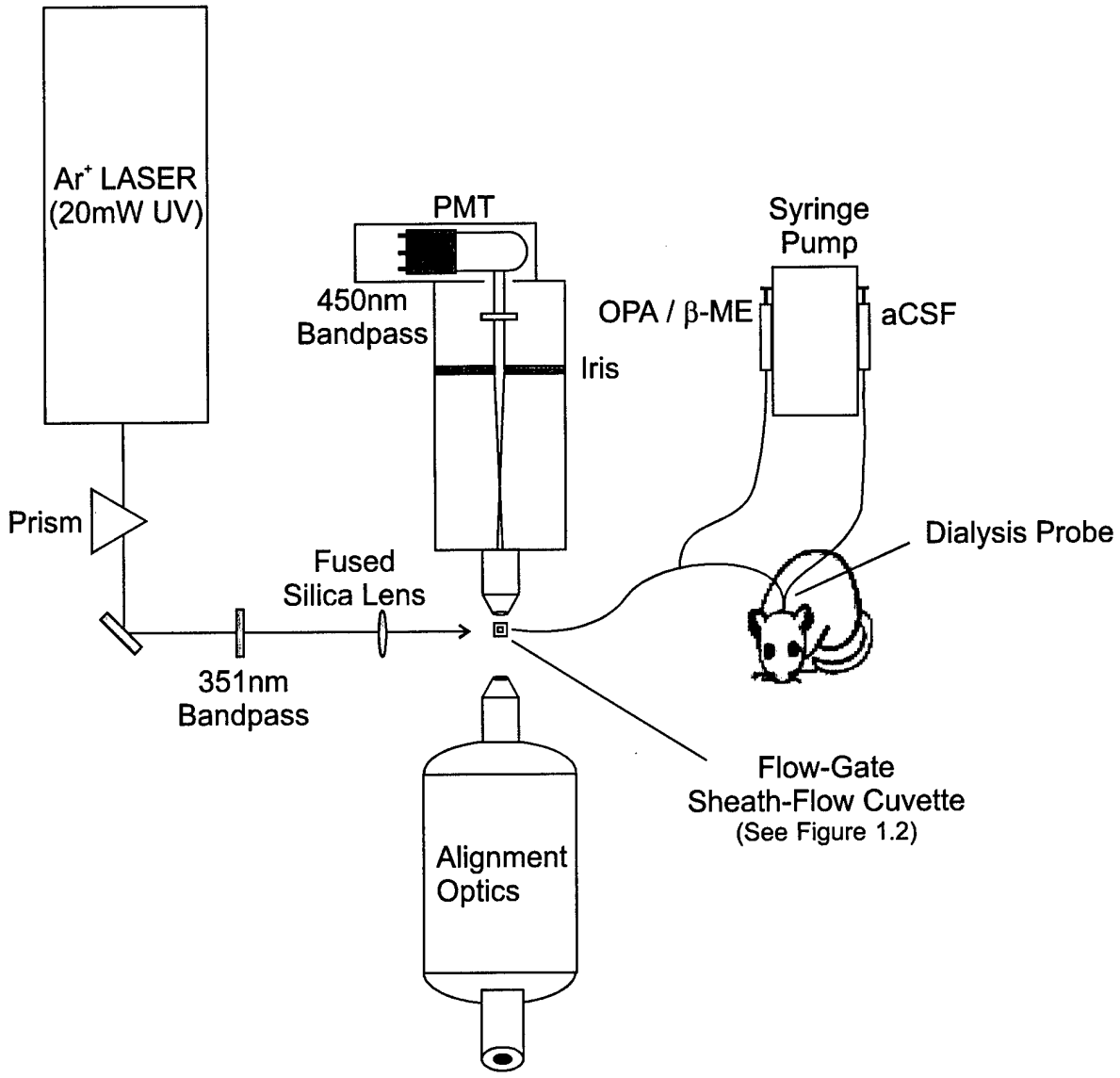


Figure 1.1 Schematic of the optical setup, microdialysis and on-line reaction

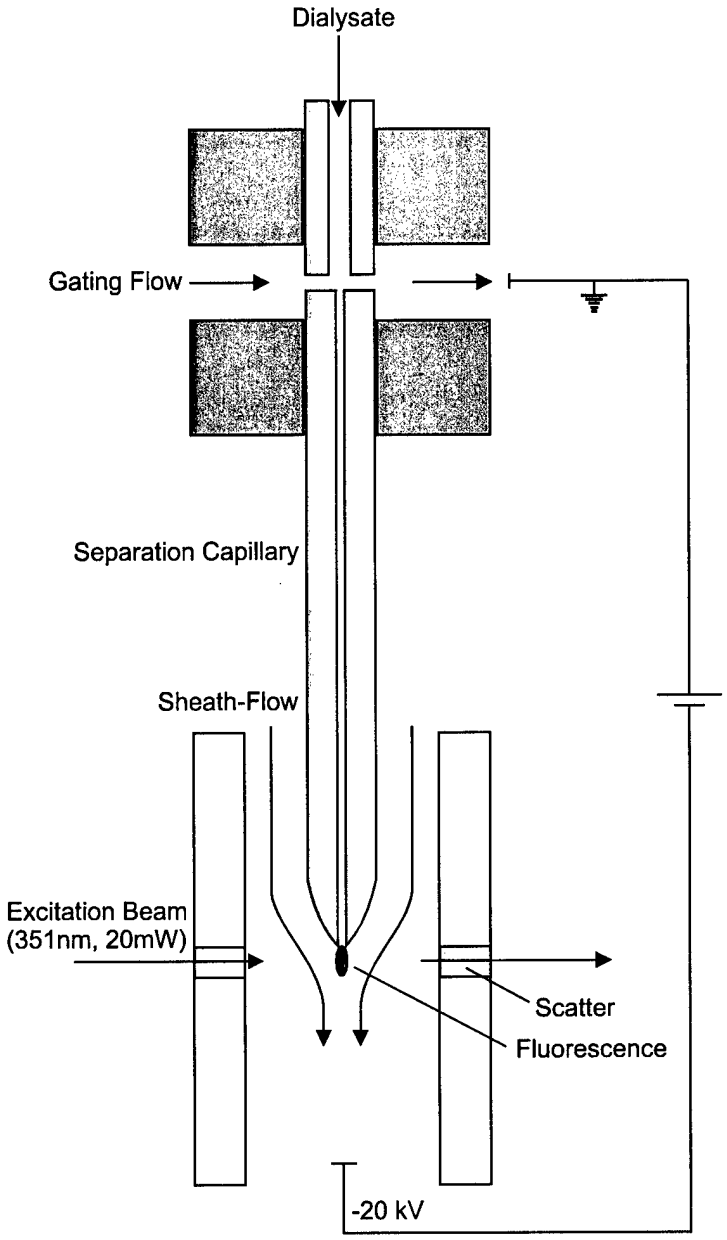
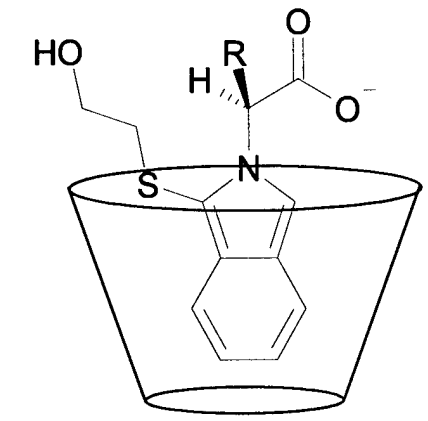
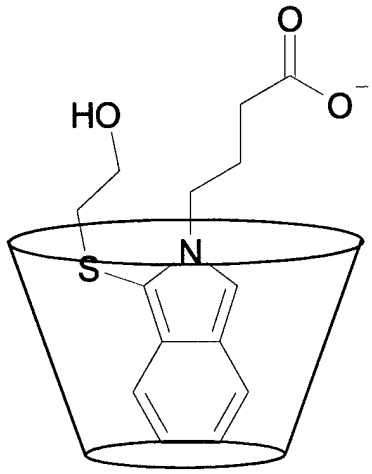


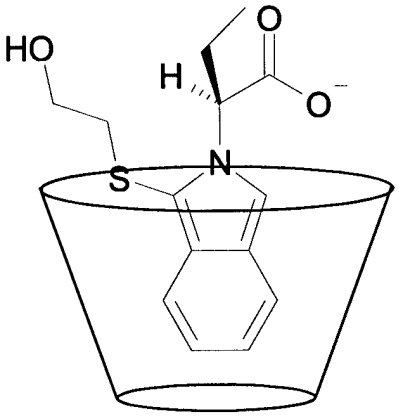
Figure 1.2 Schematic of the flow-gate interface, separation capillary and the sheath-flow cuvette.



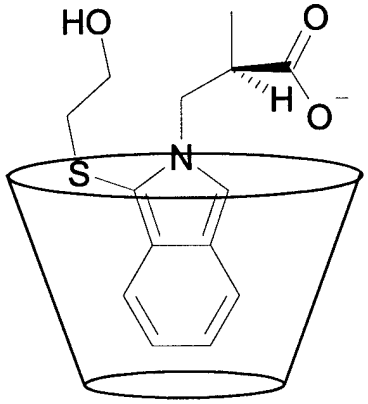
Generic Amino Acid



GABA



α-ABA



β-ABA

Figure 1.3 Diagram of the expected configurations of the isoindole - β-cylcoldextrin complexes. Based on steric considerations, the trend in binding constants is expected to be: GABA > β-ABA > α-ABA = amino acid

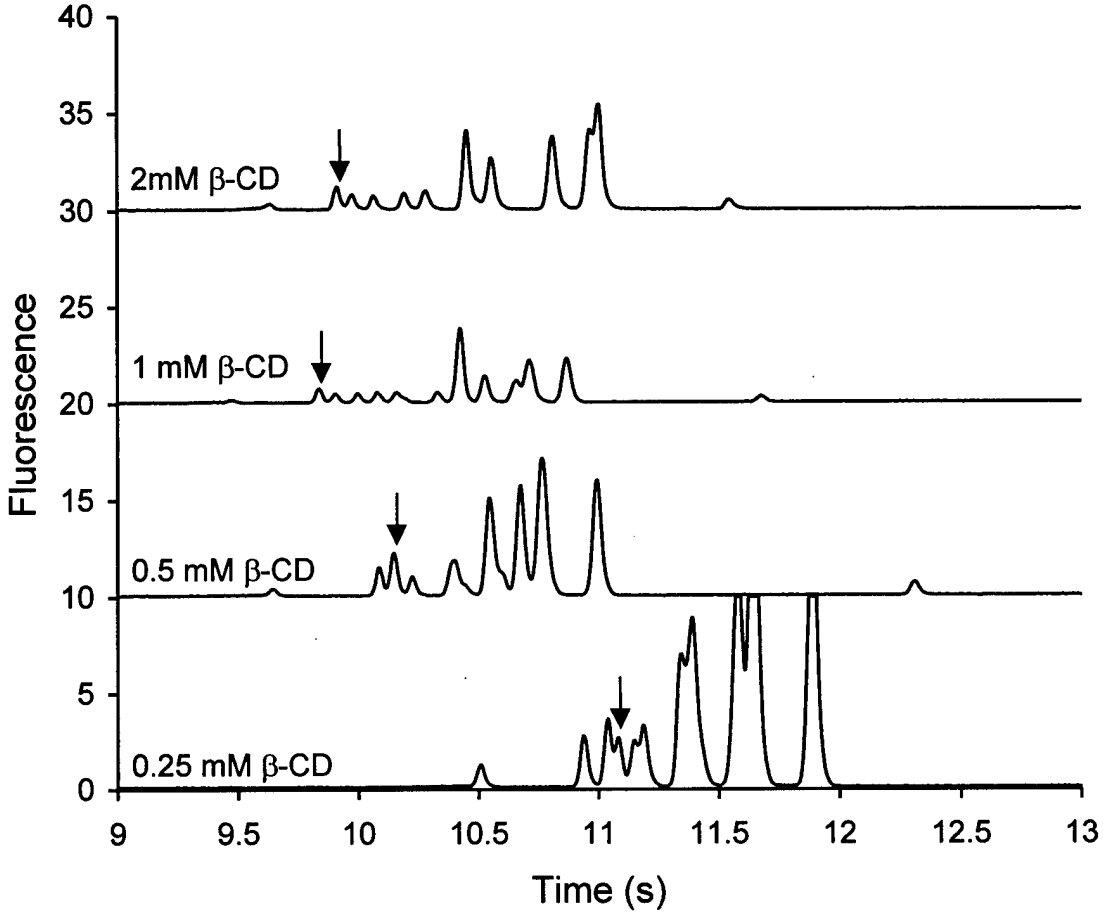


Figure 1.4 Electropherograms showing the effect of β-CD on the selectivity of the separation. The arrows indicate the migration time of GABA

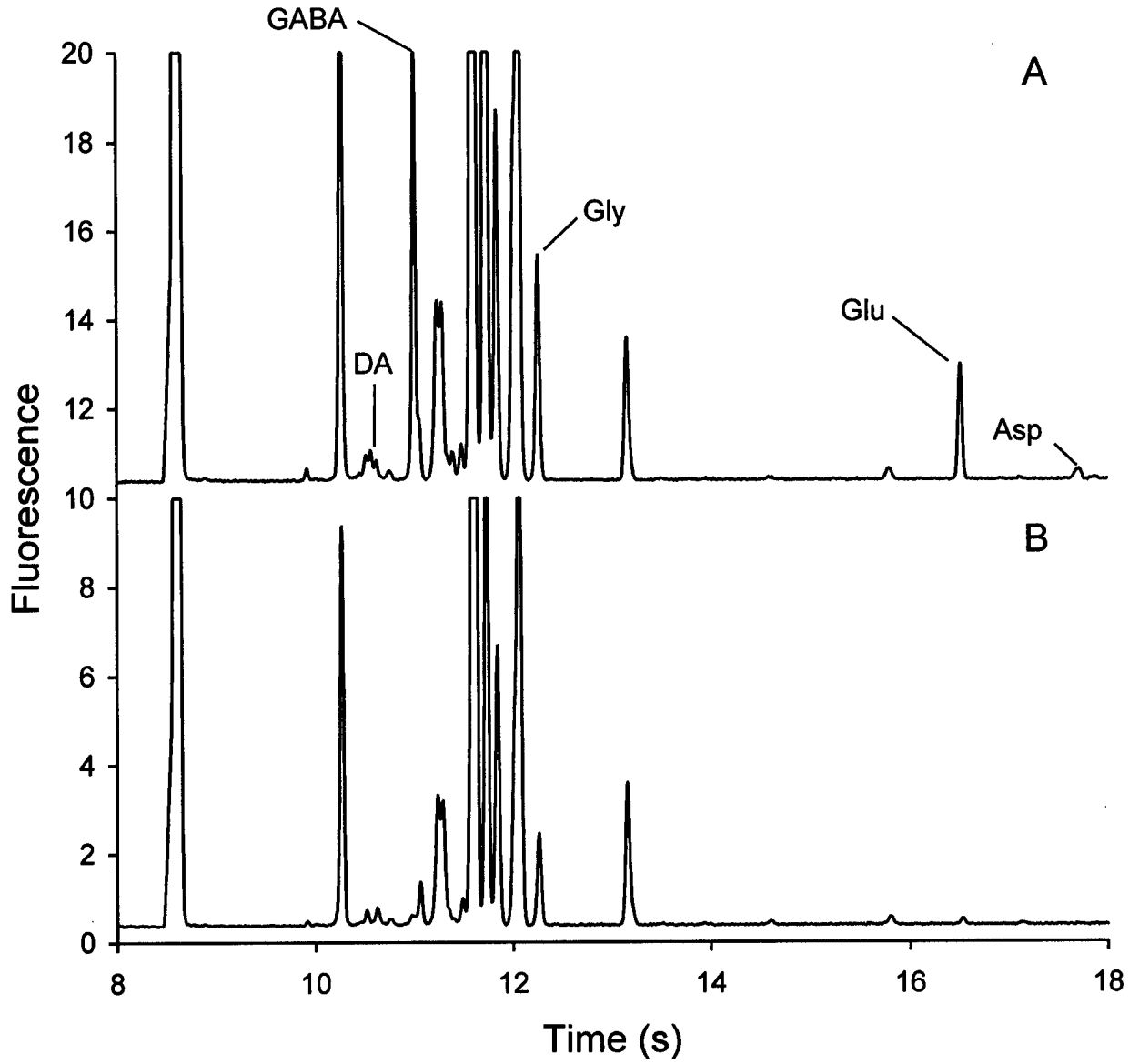


Figure 1.5 *In vivo* analysis of extracellular neurotransmitter levels in the rat striatum. Figures A and B are the stimulated (145mM KCl) and basal levels, respectively.

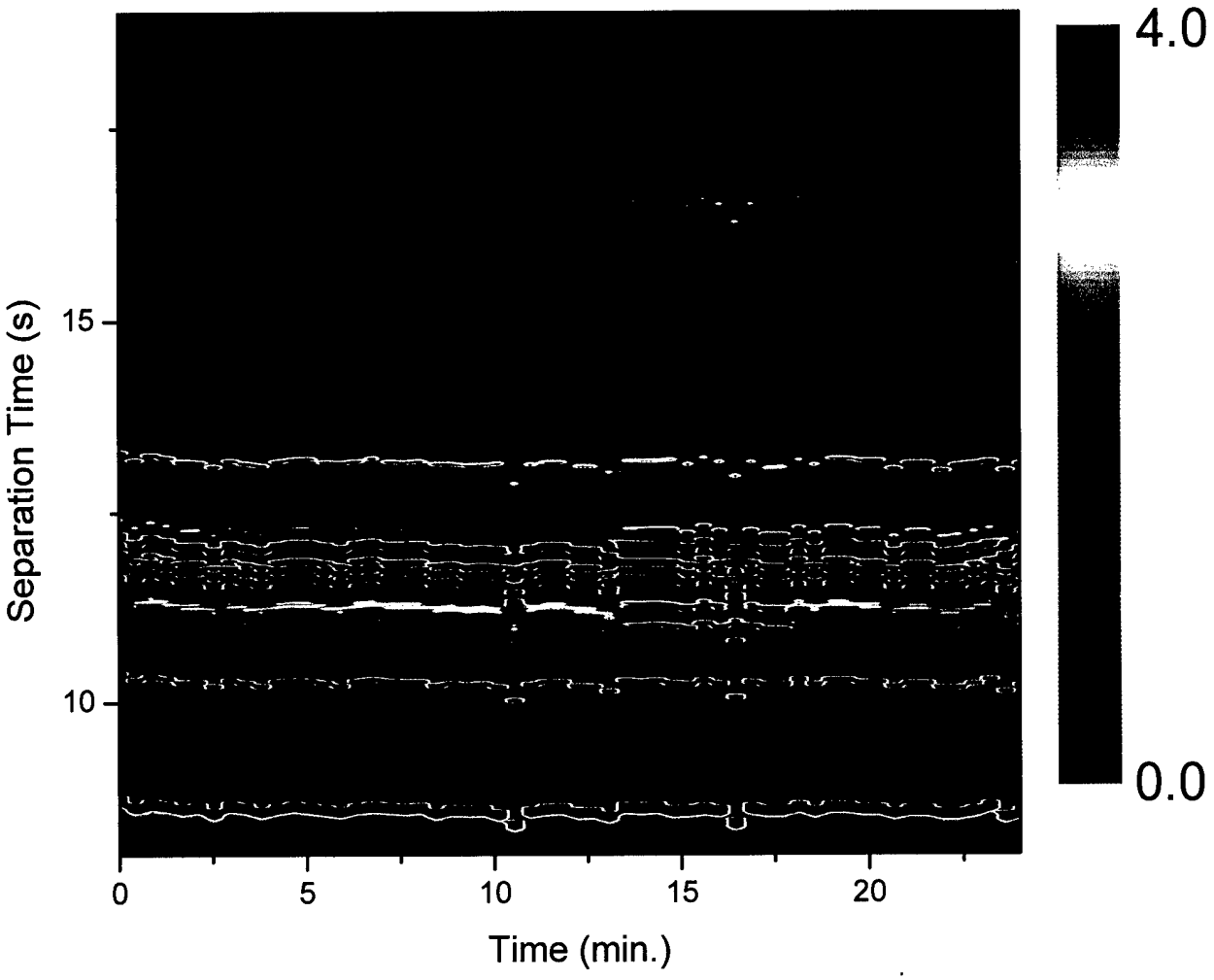


Figure 1.6 Contour plot of neurotransmitter levels during KCl (145mM) stimulation. The red line indicates the time course of the stimulation.

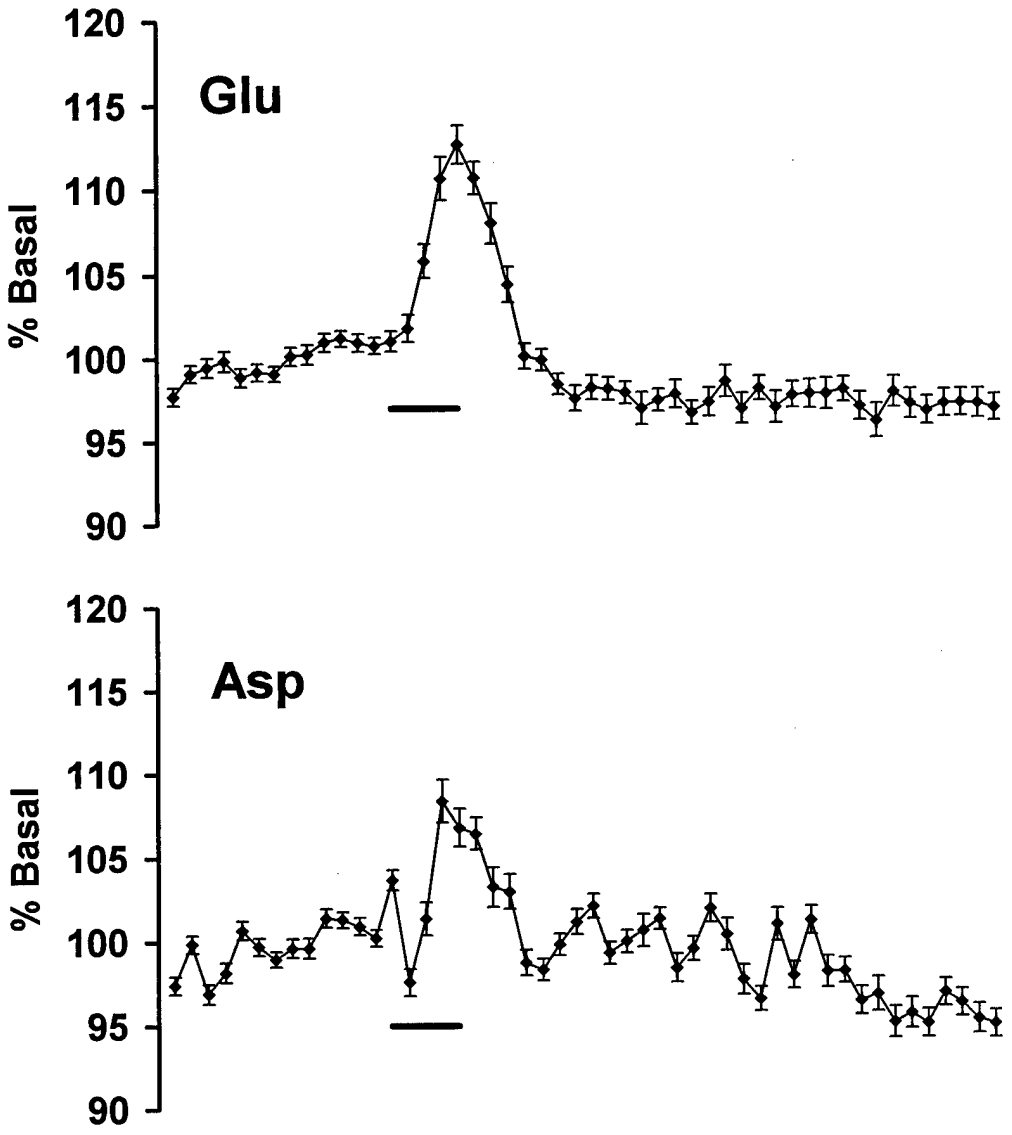


Figure 2.1 Electrical Stimulation of the Perforant Path. Bars indicate stimulation and represent 20 s. Data are presented as mean +/- SEM (21 rats, n = 80 stimulations)

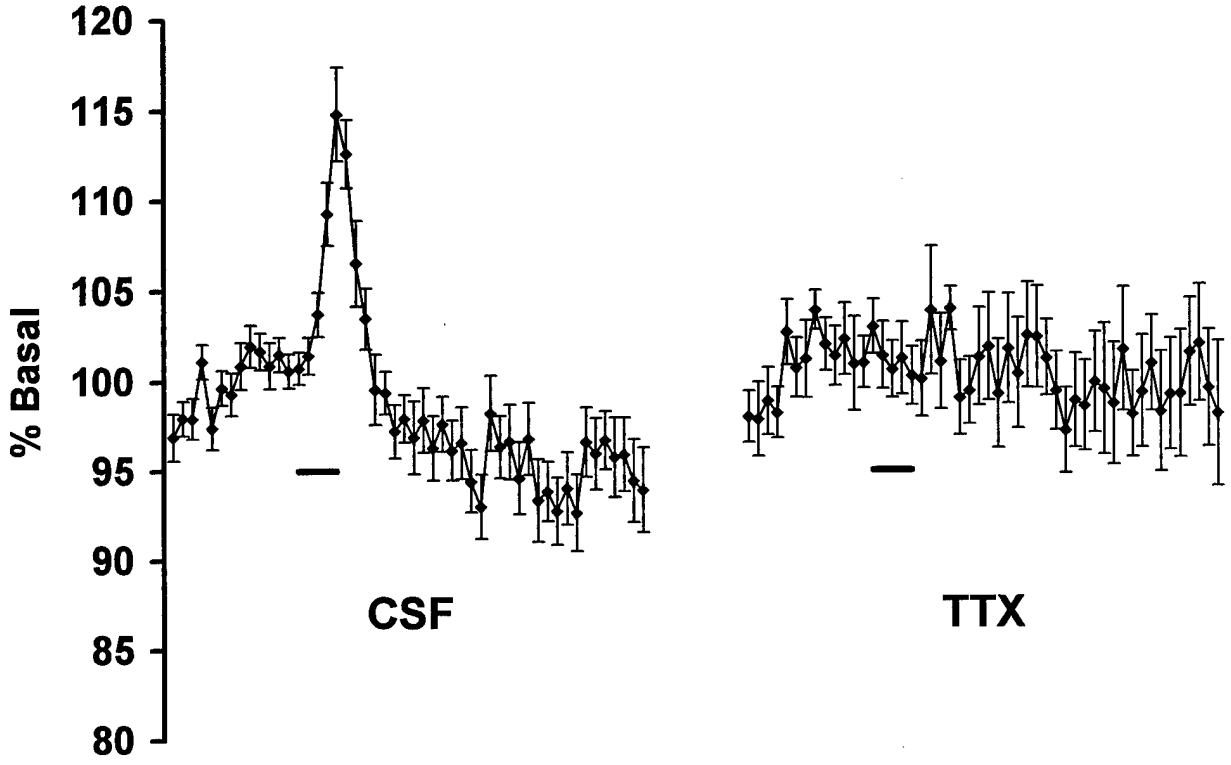


Figure 2.2 Effects of 2 μM TTX on Electrically-Stimulated GLU Release. Blockade of sodium channels completely eliminates stimulated increases in GLU. Bars indicate stimulation and correspond to 20 s. (4 rats, n = 16)

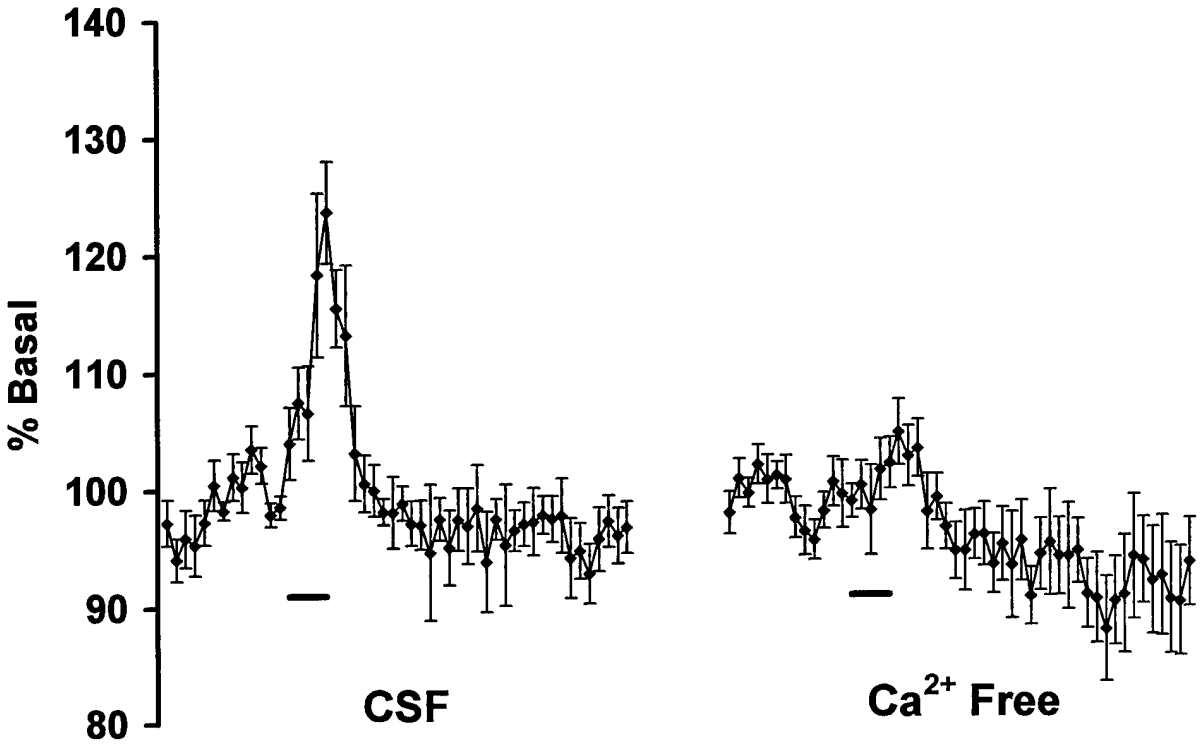


Figure 2.3 Effects of Ca2+ Depletion on Electrically-Stimulated GLU Release. Removal of Ca2+ from the perfusion medium plus addition of 2 mM EGTA reduces the stimulated overflow of GLU to 17% of control levels. Bars indicate stimulation and correspond to 20 s.

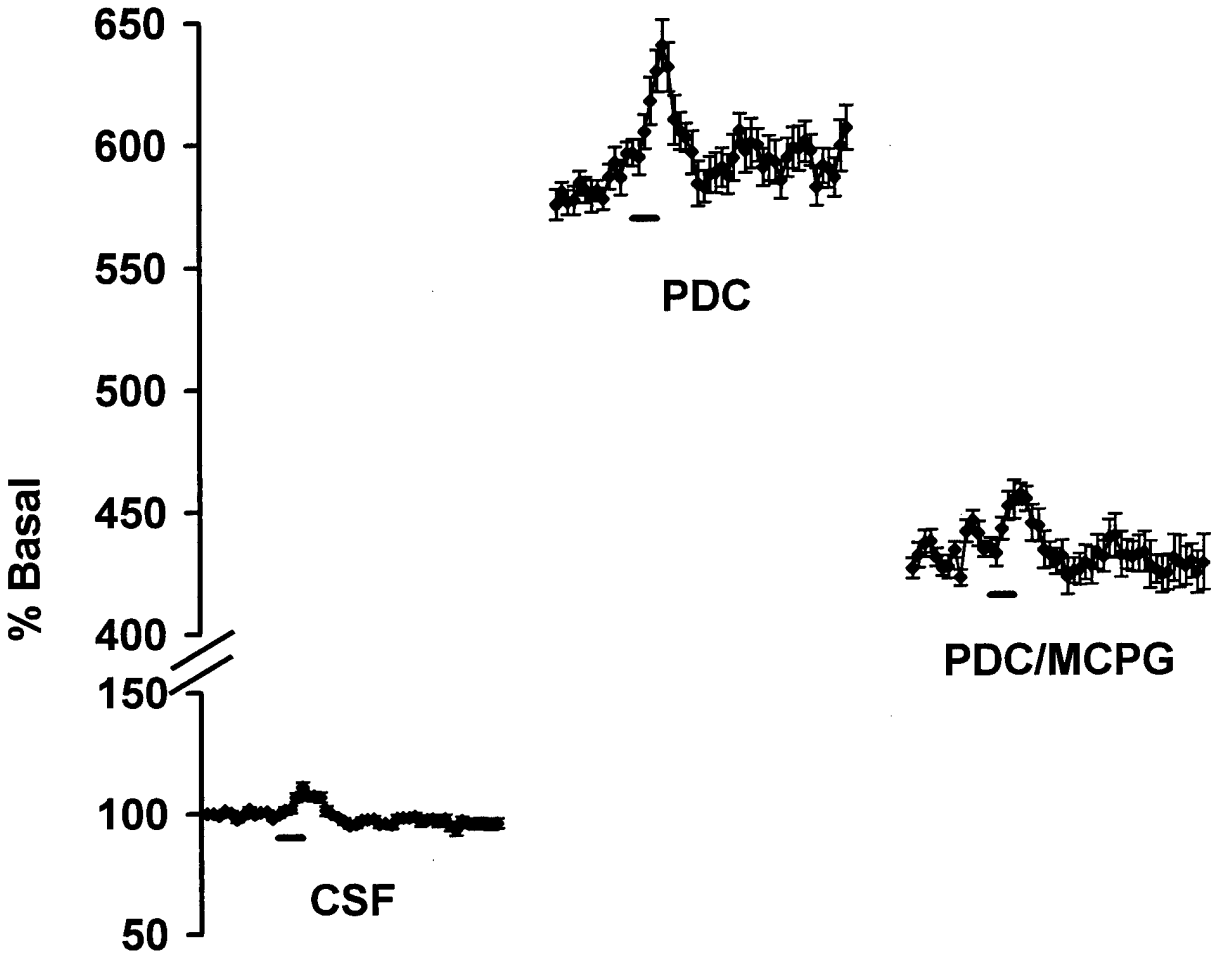


Figure 2.4 Effect of Uptake Inhibition and Receptor Blockade on Electrically-Stimulated GLU Release. Addition of 200 μM PDC to the perfusion medium results in a 584% increase in basal levels of GLU and a 987% increase in stimulated overflow. Also adding 200 μM MCPG gives only 431% and 583% increases in basal and stimulated overflow values respectively. Bars indicate stimulation and correspond to 20 s. Note break in axis. (4 rats, n = 16)

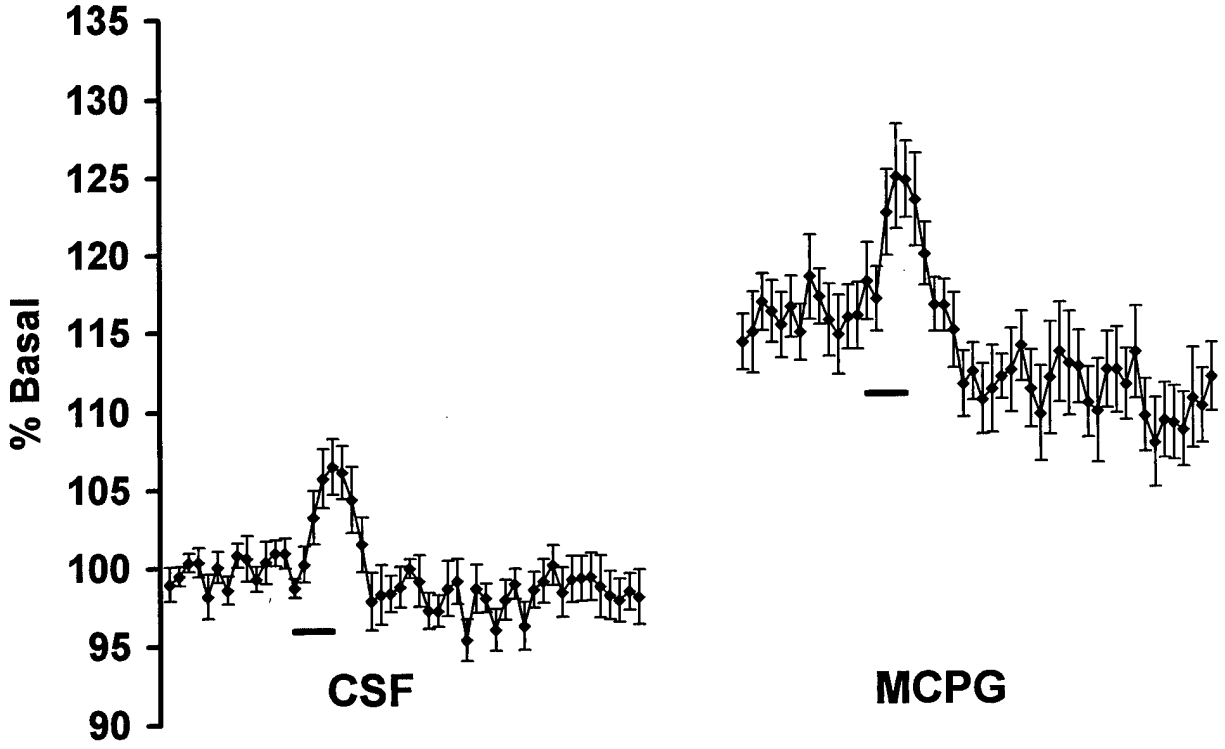


Figure 2.5 Effect of Metabotropic Receptor Blockade on Electrically-Stimulated GLU Release. Addition of 200 μM MCPG to the perfusion medium results in a 114 % increase in basal levels of GLU and a 149 % increase in stimulated overflow. Bars indicate stimulation and correspond to 20s. (3 rats, n = 14)

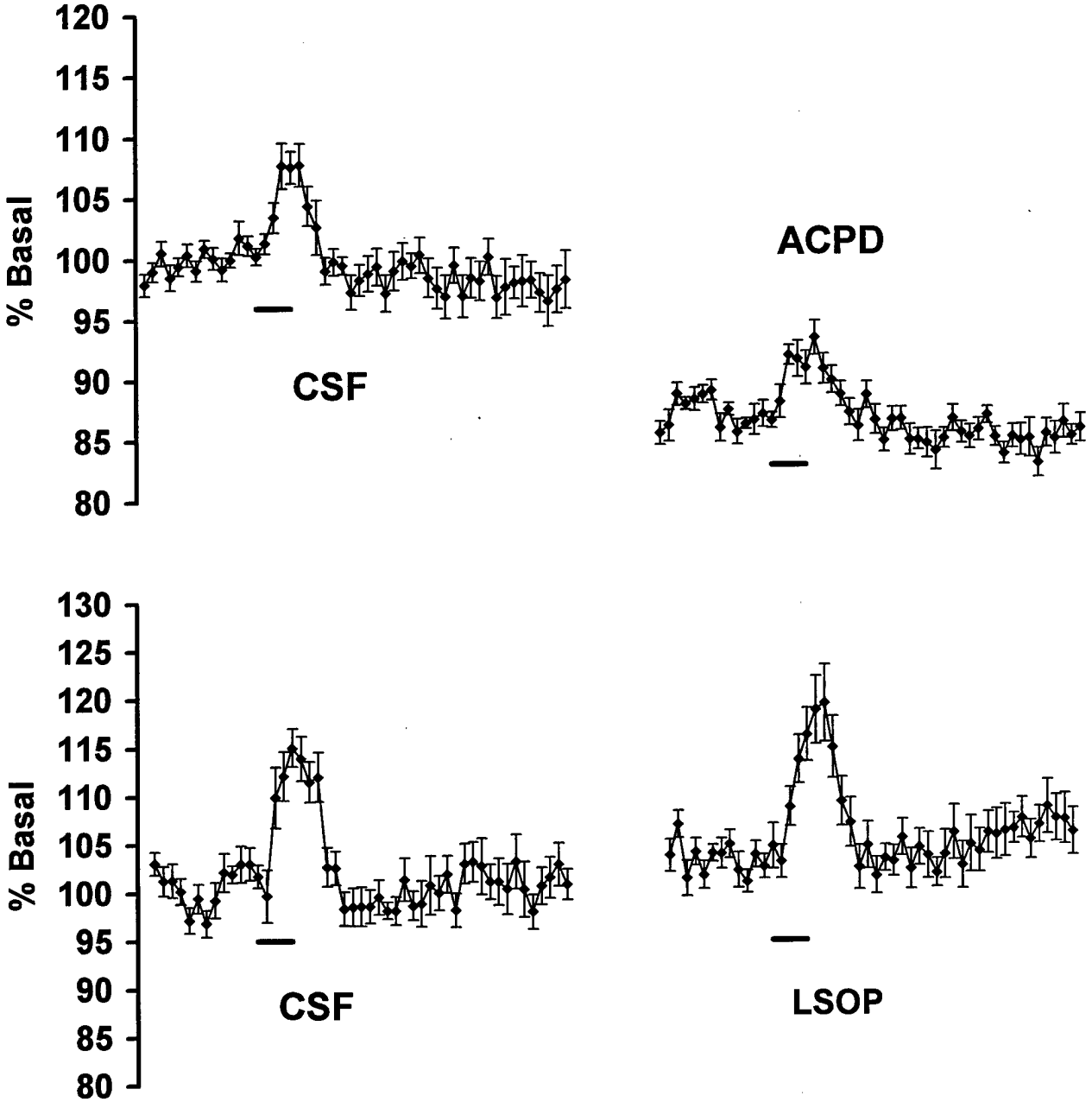


Figure 2.6 Effect of Metabotropic Receptor Activation on Electrically-Stimulated GLU Release. Addition of 200 μM ACPD to the perfusion medium results in a 13% reduction in basal levels of GLU. L-SOP (20 μM) had no significant effect. Bars indicate stimulation and correspond to 20 s. (4 rats, n =16 for each plot)

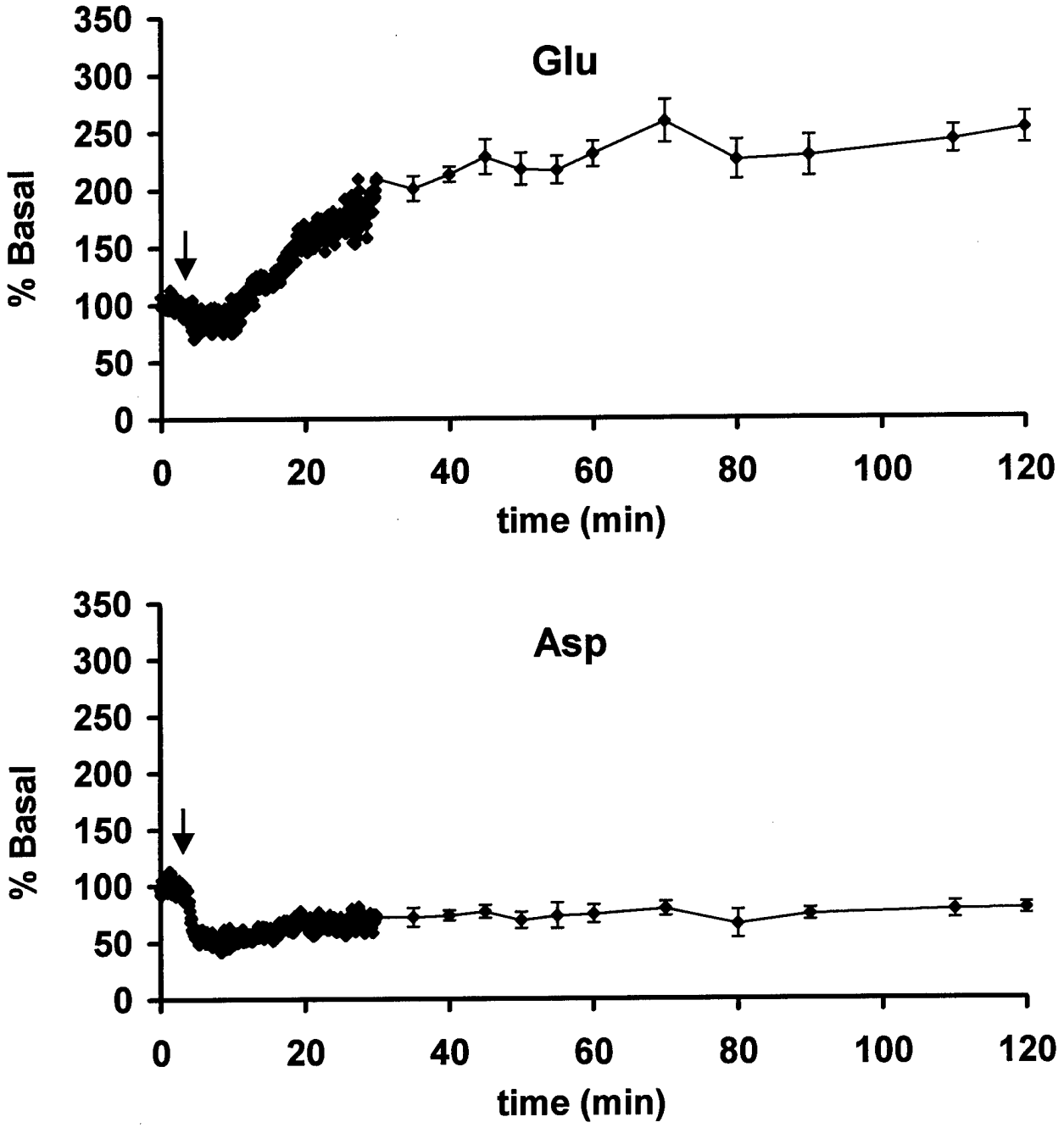


Figure 3.1 Effect of Intrahippocampal Pilocarpine Infusion on GLU and ASP Levels. Pilocarpine (10mM) was infused (see arrow) through the dialysis probe for the duration of the experiment (2 rats).

H. APPENDIX II (Reprints)

The attached abstracts are based upon results from studies that are related directly to the goals of this project. Two abstracts were presented at Eighth International Conference On *In Vivo* Methods and the third will be presented at the upcoming Annual Meeting of the Society For Neuroscience.

Capillary electrophoresis and capillary chromatography for *in vivo* monitoring of amino acid and peptide neurotransmitters

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Introduction

As a strategy for *in vivo* measurements, microdialysis excels in terms of selectivity and multi-analyte measurement capability. The weaknesses of microdialysis include difficulty with quantification, poor temporal resolution and poor spatial resolution. Numerous reports have called attention the difficulty of relating dialysate concentration to actual *in vivo* concentration due to the effects of active control of *in vivo* concentration on the relative recovery. *In vivo* calibrations have been described to circumvent this problem; however, they may be difficult to use or have limited validity. Microdialysis is also limited in terms of spatial resolution. Many brain structures, especially in experimental animals such as rodents, are too small (less than a few mm^3) to have dialysis probes implanted in them. Furthermore, within larger structures considerable heterogeneity may exist which can be hidden by the large size of the probes. Finally, the probes are large enough to cause some damage upon implantation which creates unknown effects on the results.

Temporal resolution is usually limited by mass sensitivity of the analytical method coupled to the probe. In other words, the time required to remove enough sample to be detectable by the analytical technique will determine the temporal resolution. Microdialysis sampling times are often 10 to 30 min; however, recent work has pushed this to <120 s for some neurotransmitters. This improvement has been brought about by the use of techniques with high mass sensitivity such as microbore LC and capillary electrophoresis (CE). With short sampling times and on-line coupling, these systems provide nearly real time chemical monitoring with good temporal resolution. Despite these improvements, it should be recognized that neurotransmitter concentrations change on the second time scale in the brain therefore, these measurements still distort the dynamics and prevent observation of many neurological events.

In our laboratory we have explored coupling microscale analytical techniques to microdialysis in order to ameliorate many of these limitations. The main advantage of these techniques is that they have excellent mass sensitivity, in the attomole range, which allows use of smaller probes for better spatial resolution, more frequent sampling for better temporal resolution, and lower flow rates for quantitative recovery. In this report, we will discuss advances in using capillary LC and capillary electrophoresis for *in vivo* monitoring.

Materials and Methods

Capillary Electrophoresis

The basic instrument is illustrated in figure 1. Dialysate is pumped into a tee which also has derivatization reagent pumped in through another arm of the tee. The resulting mixture flows out of the tee into a reactor capillary where the derivatization reaction takes place. For amino acid detection, the OPA/BME reagent is pumped into the tee which allows a fast fluorogenic reaction with primary amines. The dialysate is pumped into a flow-gated interface which controls periodic injection of sample into the separation capillary. Analytes are separated at 1000 to 3000 V/cm over a 3 cm capillary length. Detection is accomplished using laser-induced fluorescence (LIF).

Capillary LC

All capillary LC measurements were performed with off-line fraction collection. Columns were prepared by packing 30 cm by 50 μm i.d. fused silica capillaries with 5 μm Alltima C-18 particles using

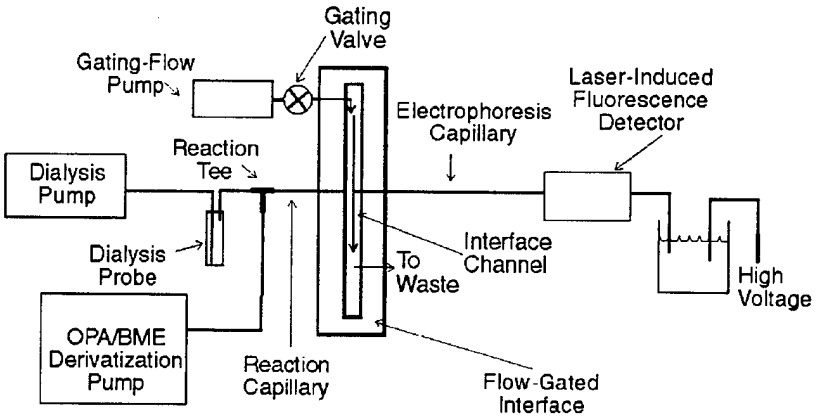

Figure 1. *Schematic of on-line microdialysis-CE instrument.*

previously described techniques. The capillary LC injection and separation system is similar to that described previously with the exception that an Isco 100 DM syringe pump (Lincoln, NB) was used for mobile phase delivery. All experiments were performed in a constant pressure mode (13.8 MPa) and volumetric flow rates were typically 0.3 μL/min. Electrochemical detection was accomplished using a 1 mm long x 9 mm diameter carbon fiber cylinder electrode, fabricated in-house, and inserted into the outlet end of the LC column. The outlet of the column was mounted in an electrochemical cell containing 100% aqueous component of mobile phase as a supporting electrolyte and fitted with an Ag/AgCl reference. Samples were derivatived using a modification of the OPA/t-BuSH derivatization reaction.

In vivo Measurements

All measurements were performed in the striatum of chloral hydrate anesthetized male Sprague-Dawley rats. Side-by-side microdialysis probes with 250 μm diameter and 2 mm active tips were prepared according the method of Justice. For slow flow rate measurements, the probes were perfused at 80 nL/min and for other measurements the rate was 1.2 μL/min. Drugs were added at the stated concentration through the dialysis probes. Electrical stimulations of the corticostriatal pathway began 30 minutes after drug administration and consisted of 0.5 ms square wave depolarizing pulses (80 mA) applied at 20 Hz for 10 s using a S5 Stimulator (Grass Medical Instruments, Quincy, MA). The stimulus frequency was based upon the capacity of corticostriatal neurons to follow high-frequency burst firing patterns and is similar to stimuli used by other investigators to activate corticostriatal neurons. For multiple stimulations on one animal, 30 min was allowed between stimulations. Preliminary studies showed that this time allowed reproducible evoked responses to be obtained without apparent fatigue. All experimental uses of laboratory rats in the present study were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conform with policies and procedures set forth by U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Results and Discussion

Electrically stimulated overflow of GLU and ASP detected by CE

The speed and resolution of the separation allows GLU and ASP to detected at 5-s intervals on a routine basis. In some experiments, we have been able to resolve several other compounds including GLY on this time scale. Electrical stimulation of the prefrontal cortex results in a rapid and brief elevation of GLU and ASP levels during the course of stimulation (10-20 s) followed by a decay that

appears to be controlled by re-uptake. The GLU and ASP release appear to by of neuronal origin since they are TTX-sensitive and Ca^{2+}-dependent even though measurement of basal levels gave ambiguous results. Detection of neurotransmission in this way has allowed characterization of the effects of uptake inhibitor, metabotropic glutamate receptor, and ascorbic acid regulation of GLU/ASP.

Direct sampling probe with CE

While the good mass sensitivity of CE allows considerable improvements in the monitoring possible by microdialysis, further improvements may be limited by the probe itself. Therefore, we have begun to explore direct sampling of the extracellular fluid of the brain and on-line analysis by CE. In this system, electroosmotic flow is used to remove fluid at a rate less than 10 nL/min through a fused silica capillary that is 10 μm i.d. and 25-50 μm outer diameter at the tip. Samples are derivatized and analyzed in a fashion similar to that described for the microdialysis measurement. Using this experimental protocol, we have been able to collect samples for over 1 h and analyze the extracellular fluid. This approach offers much higher spatial resolution and direct quantification of the extracellular fluid compared to microdialysis. At present however, the sensitivity and reliability of this approach needs to be improved for in vivo measurements.

10 sec fraction collection for monitoring all amino acid neurotransmitters

Capillary LC has similar mass sensitivity to CE and offers the opportunity to separate more neurotransmitters in a single step; however, at present the separations are slower and not amenable to high temporal resolution in an on-line experiment. Given these considerations, we have performed preliminary experiments to test the feasibility of collecting 200 nL fractions from microdialysis (10-s fractions at 1.2 μL/min) and measuring the primary amines by capillary LC. We were able to quantify 14 amino acids in these fractions. Using K^+ stimulation we detected dynamic changes in ASP, GLU, taurine, GABA, and GLY levels. In addition, we demonstrated selective enhancement of GABA levels in response to nipocotic acid, a GABA uptake inhibitor. With appropriate automation, this technique should become a useful complement to the on-line CE measurements for monitoring a variety of compounds *in vivo*.

ANNUAL REPORT: 10/01/98 – 09/30/99
AWARD NUMBER: DAMD17-98-1-8617

Monitoring neurotransmitter amino acids by microdialysis with on-line flow gated capillary electrophoresis

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Introduction

Synaptic transmission of a neurological signal requires the release and reception of neurotransmitters. The majority of these molecules diffuses into the extracellular space and can be measured before they are absorbed into the surrounding tissue. This process occurs on a fairly fast time scale (seconds) and involves fmols of material. Therefore, a fast, sensitive analytical method is required. Cyclic voltammetry has been used extensively to study dopamine while selective electrodes measure glutamate (GLU) with good results. These methods do not however allow more than one component to be monitored at a time. In order to study multiple analytes, extracellular fluid can be sampled by microdialysis, fractions collected, and then separated by HPLC or CZE. The temporal resolution of these methods usually is dependent on volume requirements and mass limits of detection (LOD) of the analysis. On-line methods remove most of the volume requirements but are still limited by LODs and additionally separation time.

We have previously coupled microdialysis to a flow gated capillary electrophoresis system that is capable of 5 s separations of GLU and aspartate (ASP) while providing temporal resolution of 7 to 14 s.[1,2] Recent improvements have made separation the limiting step with respect to temporal resolution which can be as fast as 3.2 s. This system has temporal resolution approaching glutamate selective electrodes while at the same time allows for multicomponent analyses. Besides GLU and ASP, additional peaks in the basal electropherogram have been identified as arginine, glycine (GLY), reduced glutathione, and o-phosphoethanolamine. Interactions of excitatory and inhibitory neurotransmitter amino acids were studied by microinjection of exogenous neurotransmitter. Additionally, electrical stimulations in the striatum and hippocampus have been monitored.

Materials and Methods

Microdialysis probes (200 μm o.d., 1 or 2 mm tips) were constructed by a previously described method[3] and perfused at a flow rate of 0.60 μl/min. Probes were implanted in chloral hydrate anesthetized male Sprague-Dawley rats (250-300 g) in the dentate gyrus of the hippocampus based on stereotaxic coordinates. Microdialysate was then mixed with the fluorogenic reagents o-phthaldehyde/β-mercaptoethanol via a tee and allowed to react for approximately 50 s before injection at the flow gated interface. Briefly, the reaction and separation capillaries are approximately 30 μm apart while a cross flow of electrophoresis buffer sweeps away dialysate before it can reach the inlet of the separation capillary. To inject, the cross flow is stopped for a set length of time (typically 60 ms) allowing a small plug to enter the capillary.

Electrophoresis is carried out on a 3.5 cm to detector (5 cm total) 10 μm i.d. capillary at E = 2400 V/cm. Analytes are detected fluorescently after excitation by the 354 nm line of a He-Cd laser. Stimulation of the perforant pathway in the hippocampus utilized 0.1 ms, 20 V square wave pulses at 20 Hz for 20 s with a concentric bipolar electrode. 40 pmol of a neurotransmitter were injected using a 26 ga. syringe (100 μm from the probe).

Results and Discussion

Introduction of exogenous GLU to the hippocampus results in its immediate increase in microdialysate concentration as expected and an increase in both ASP and GLY. On average, 280 fmol of

GLU is collected after a 40 pmol injection. ASP increases by 170% with 0.9 fmol collected while GLY increases slightly but is difficult to quantify because it is not completely resolved from other components in the electropherogram. The increase in ASP is reasonable as it is an excitatory amino acid and performs many of the same neurological functions as GLU. The increase in GLY is interesting because its neurological effects are inhibitory. The exact nature of the response has yet to be determined.

Preliminary results of electrical stimulation of the perforant pathway and measurement of GLU and ASP in the dentate gyrus show a small but reproducible increase of both amino acids. Comparison of response to striatal studies indicates a much more rapid uptake of neurotransmitters in the hippocampus.

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MONITORING AMINO ACID NEUROTRANSMITTERS IN VIVO WITH HIGH TEMPORAL RESOLUTION USING MICRODIALYSIS. R.T. Kennedy[1], T. Vickroy, S.T. Witowski, J.E. Thompson, B. Boyd, and I. Phillips[2]*, [1]Dept. of Chemistry, [2]Dept. of Physiology, Univ. of Florida, Gainesville, FL 32611.

As a strategy for in vivo measurements, microdialysis excels in terms of selectivity and multi-analyte measurement capability; however, microdialysis is hampered by several weaknesses including poor temporal and spatial resolution. Coupling microdialysis to capillary electrophoresis (CE) and capillary liquid chromatography (LC) is a way to ameliorate these limitations. These techniques offer mass sensitivity in the attomole range which allows use of smaller probes for better spatial resolution, more frequent sampling for better temporal resolution, and lower flow rates for quantitative recovery. We have built two systems for monitoring neurotransmitters in vivo that take advantage of capillary separation methods. In the first, a microdialysis probe is coupled directly to a CE capillary on-line. Using this system we have: 1) detected and characterized electrically stimulated overflow of glutamate and aspartate in rat striatum and hippocampus, 2) demonstrated regulation by metabotropic glutamate receptors and uptake transporters, and 3) tested the hypothesis that ascorbate can affect glutamate release. As a second route to improved monitoring, we have coupled microdialysis off-line with capillary LC. The high sensitivity and resolution of the LC method has allowed 16 amines to be monitored with 10 s temporal resolution. Supported by NIH and DOD.